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Removal of Atrazine Contamination in Soil and Liquid Systems Using Bioaugmentation

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Abstract: Reported levels of atrazine in soils at pesticide mix-load sites can vary between $7{\cdot}9 \times 10^{-5}$ mM and 1·9 mM. We report on a mixed microbial culture, capable of degrading concentrations of atrazine in excess of 1·9 mM. At initial concentrations of 0·046 M and 0·23 M, the mixed population degraded 78% and 21% of atrazine in soil (100 days), respectively. At the same initial concentrations in liquid cultures, 90% and 56% of the atrazine was degraded (80 days), respectively. Decreased degradation in soil samples may have resulted from atrazine sorption to soil surfaces or decreased contact between the population and the herbicide. In the 0·23 M system, we attribute incomplete degradation to phosphorous depletion. Data for carbon dioxide evolution was fitted to a three-half-order regression model, but we feel that there are limitations of the application of this model to atrazine degradation. The population uses the herbicide as a nitrogen source and little carbon is incorporated into biomass, as the energy status of carbons in the ring leads to their direct evolution as [^{14}C]carbon dioxide. This situation contributes to an evolution pattern that, when fitted to the three-half-order model, results in underestimation of the biomass produced. Data from our study suggest that our mixed culture could be used for bioremediation of atrazine at concentrations up to and exceeding those currently reported for agrochemical mixing-loading facilities.

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1 INTRODUCTION

Atrazine, a widely used herbicide that controls broadleaf and grassy weeds in corn, sorghum, sugarcane and rangeland, has been detected in ground waters, possibly as a result of normal agricultural use.[1–3] However, more serious pesticide contamination has been detected at point sources such as agrochemical mixing-loading facilities.[4] Goetsch *et al.*[5] found that atrazine in water samples from four agrochemical sites in Illinois frequently exceeded the established maximum permitted contaminant levels. Studies of agrochemical mixing-loading facilities in Illinois showed atrazine to occur at levels between $7{\cdot}9 \times 10^{-5}$ mM and 1·9 mM (mmole kg^{-1} soil) in the surface gravel/fill layer.[6] Microbial systems have been proposed as a cost-effective method for cleanup of pesticide waste.[7]

Bioremediation is an established approach for cleaning up contaminated soils and waters. Forsyth *et al.*[8]

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suggest three types of bioremediation systems: (1) solid-phase, (2) slurry-phase, (3) *in-situ* /*ex-situ* systems for cleanup of contaminated soil and ground water. *In-situ* and slurry-phase (*ex-situ*) bioremediation have been suggested for soils and ground water contaminated with atrazine.[9] *Ex-situ* remediation, such as solid-phase bioremediation, coupled with *in-situ* bioremediation has also been suggested for atrazine-contaminated soils.[4]

Successful bioremediation of dinoseb-contaminated soils, based on biostimulation of native populations, has been reported.[4,10] An alternative technique to biostimulation of native micro-organisms is bioaugmentation, the introduction of micro-organisms capable of breaking down target pesticides. Bioaugmentation could be applied as both *in-situ* and *ex-situ* remediation systems; however, in the *ex-situ* system, introduced micro-organisms can be thoroughly mixed into the contaminant matrix. Shin and Crawford[11] reported that TNT (2,4,6-trinitrotoluene)-contaminated soil could be bioremediated when the contaminated soil was treated with *Clostridium bifermentans* in a bioreactor. *Arthrobacter* (strain ATCC 33790) was used to augment PCP (pentachlorophenol)-contaminated soil, resulting in mineralization of 73 μg g^{-1} PCP.[12] Baud-Grasset and Vogel[13] used 2,4-D-degrading organisms to biodegrade over 95% of 2,4-D from sandy soils contaminated with 710 mg kg^{-1} 2,4-D.

The potential of using bioaugmentation of atrazine-contaminated soils and waters for bioremediation is great. Single organisms or mixed cultures that transform or mineralize atrazine have been isolated.[14–21] However, elevated concentrations of pesticides can greatly decrease the rate of biodegradation,[22] and there are few reports of organisms able to degrade atrazine at concentrations typical of contaminated soils at agrochemical mixing-loading facilities. The mixed culture of micro-organisms reported by Assaf and Turco[18] shows promise for use in bioremediation systems for atrazine concentrations typical of these sites.

The mixed culture of Assaf and Turco[18] was isolated from soil with a history of atrazine application. With culture optimization, the mixed culture could mineralize 96% of 0·56 mM atrazine in liquid,[18] which is in contrast with other previously reported isolates capable of only biotransformation.[14–16,19] This mixed culture mineralized atrazine at higher concentrations than other reported isolates and mixed cultures in a liquid culture, except those reported by Yanze-Kontchou and Gschwind.[20] It was also able to mineralize 87% of 0·14 mM atrazine in soil.[18] Two previous studies have shown mineralization of atrazine ($4{\cdot}6 \times 10^{-4}$ mM and 0·023 M, respectively) in soil samples.[23,24] The ability of our mixed culture to mineralize atrazine in the soil environment indicates its possible use for bioremediation of contaminated soils, without production of persistent atrazine metabolites.

The objective of this research was to determine if a previously reported mixed microbial culture could mineralize concentrations of atrazine exceeding those that might occur at agrochemical mixing-loading facilities.[18] Moreover, our study was designed to evaluate the mixed culture's ability to degrade atrazine concentrations under conditions representing both *ex-situ* bioremediation through biopiling (solid-phase bioremediation) and as slurry-phase bioremediation of waters contaminated with atrazine, such as equipment rinse water from mixing-loading facilities.

2 MATERIALS AND METHODS

2.1 Herbicide

Atrazine was used as a formulated 480 g $litre^{-1}$ SL (Aatrex 4L; Ciba Geigy, Greensboro, NC) to facilitate excess loading rates and to simulate contaminant material found at agrochemical facilities. Atrazine was applied to soil (0·0014, 0·046, 0·23, 0·46 and 1·39 mole AI kg^{-1} soil) and at 0·0014, 0·046, 0·23, 0·46 and 1·39 M (AI) in liquid samples. [*U-ring*-^{14}C]atrazine (specific activity, 4·5 Mbq $mmol^{-1}$, Sigma Chemicals, St. Louis, MO) was mixed with formulated atrazine to achieve a target level of 16·7 Bq g^{-1} of soil (Bq ml^{-1} liquid culture), and mixed for 24 h prior to use. [^{14}C]Atrazine was also mixed with technical grade atrazine, 99% purity (ChemService, West Chester, PA), for mixed-culture viability studies.

2.2 Soil

A Drummer soil (silty clay loam, 4% organic matter, pH 6·9) was used. Soil samples were obtained from the upper 10 cm of conventionally tilled plots planted to corn for over 18 years. The site on the Purdue Agriculture Experiment Station, West Lafayette, IN, had been treated with atrazine at rates recommended for weed control. Soil was sieved through a 2-mm mesh screen and refrigerated (4°C) at field moisture conditions. Moisture content was corrected to −0·01 MPa before use.

2.3 Liquid media

Enrichment of the mixed culture capable of degrading atrazine was previously reported.[18] Liquid cultures were prepared in minimal basal salts medium supplemented with 5 g $litre^{-1}$ glycerol (carbon source) and

atrazine at 0·23 mM (nitrogen source). The minimal basal salts medium was composed of K_2HPO_4 and KH_2PO_4 (0·1 M; pH 7·0), $MgSO_4$ (0·8 mM), $CaCl_2$ (0·18 mM), $MnCl_2$ (5 μM), $CuCl_2$ (1 μM), $FeCl_3$ (1 μM), $CoCl_2$ (1 μM), $NiSO_4$ $6H_2O$ (1 μM), $Al_2(SO_4)_3$ $18H_2O$ (1 μM), H_3BO_3 (1 μM), $FeSO_4$ (1 μM) and Na_2MoO_4 $2H_2O$ (8 μM).

2.4 Cultures

Cultures were grown until mid-log phase in the minimal basal salts medium + 5 g litre^{-1} glycerol supplemented with atrazine. For samples representing slurry-phase remediation of atrazine-contaminated waters, formulated atrazine amended with [^{14}C]atrazine was added directly to the mid-log phase cultures. For soil samples, cultures were centrifuged at 2000*g* for 0·3 h (Sorvall Model RC-2, E.I. du Pont de Nemours & Co., Inc., Chicago, IL) to sediment micro-organisms. Approximately 60% of the supernatant was then decanted and micro-organisms resuspended in the remaining supernatant to inoculate soil samples.

Microbial populations in liquid cultures and concentrated cultures for soil inoculation were determined by direct count, utilizing acridine orange staining (0·3 g litre^{-1} in $NaHCO_3$) of microbial cells. Cells were counted by epifluorescence microscopy (Standard Microscope 16, Zeiss, West Germany,) under UV light (Zeiss, West Germany). Soil samples were inoculated with the mixed culture at approximately $4{\cdot}7 \times 10^7$ cells g^{-1} soil. The mixed culture population in aqueous samples was $5{\cdot}1 \times 10^8$ cells ml^{-1}.

2.5 Soil sample preparation

Soil samples representing *ex-situ* biopiling were prepared in 250 ml glass vessels with 15 g (dry soil equivalent basis) of soil per jar. Samples were then inoculated with the mixed culture and mixed, unless stated. Water input from mixed culture inoculation was accounted for when adjusting soil samples to $-0{\cdot}01$ MPa. Formulated atrazine amended with labeled atrazine was added to the soil samples and incorporated. Sample vessels were sealed with rubber stoppers fitted with sodium hydroxide traps for evolved carbon dioxide and tubing to allow oxygen recharge through the sodium hydroxide. In initial 1·39 mole kg^{-1}-soil and 0·46 mole kg^{-1}-soil atrazine soil studies, the air was exchanged into sample containers when sodium hydroxide traps were exchanged. Subsequent experiments in soil were conducted under oxygen pressure (5 mm Hg) to enhance oxygen recharge and were maintained at 22($\pm$2)°C. Additional samples were prepared, with no inoculum added, to determine degradative contributions of native microbial populations and chemical degradation. Evolution of carbon dioxide due to native soil micro-organisms or chemical degradation was not observed. Samples were prepared in triplicate.

Traps for measuring evolved carbon dioxide were filled with sodium hydroxide (1 M; 5 ml). Stoppered sample vessels were opened at four-day intervals to exchange carbon dioxide traps and allow air exchange. Samples of trap solution (0·5 ml) were removed and placed in scintillation cocktail (Cytoscint™, ICN Biochemicals, Inc., Costa Mesa, CA). The scintillation samples were counted by liquid scintillation (Tri-Carb 1600 TR, Packard Instruments, Downers Grove, IL) and quantified against external standards.

2.6 Liquid sample preparation

Cultures were prepared in 50-ml volumes in 125-ml Erlenmeyer flasks plugged with cotton, and maintained at 22($\pm$2)°C on a rotary shaker at 150 rev min^{-1}. Formulated atrazine amended with [^{14}C]atrazine was then added to the cultures at both 0·046 M and 0·23 M concentrations, which were returned to the shaker to enhance aeration and atrazine–micro-organism contact. Sterile liquid samples were also prepared in minimal basal salts medium at each atrazine concentration, to determine the amount of ^{14}C evolution caused by chemical degradation.

As with soil samples, there was no measurable ^{14}C evolved from sterile (uninoculated) samples, indicating no detectable chemical degradation of added atrazine. Liquid samples were prepared in triplicate and were continuously shaken between sampling times.

At four-day intervals, 0·2-ml samples of the culture were removed and placed in scintillation cocktail. Scintillation samples were counted by the same procedure as sodium hydroxide trap samples from soil samples. The [^{14}C]atrazine remaining in the system was measured and ^{14}C evolved was calculated by difference. Final atrazine concentrations remaining in liquid samples were also determined by high pressure liquid chromatography (HPLC).

2.7 Use of HPLC

The mixed cultures grown to mid-log phase were confirmed in their ability to degrade atrazine (50 μg ml^{-1}) prior to use, using HPLC (Varian Model 5000 Liquid Chromatograph, Varian Instrument Group, Walnut Creek, CA). The HPLC was equipped with a Gilson KM Holochrome UV-VIS detector at 230 nm. Separation of atrazine and hydroxyatrazine (if present as this was the only metabolite detected as a result of degradation by this mixed culture), was achieved with a $250 \times 4{\cdot}6$ mm reverse phase C_{18} column (Spherisorb 10 ODS 2, Phenomenex, Torrance, CA). Samples were analyzed at 22($\pm$2)°C, with a constant 1 ml min^{-1} flow

rate of methanol + water (60 + 40 by volume), adjusted to pH 7·4 with sodium hydroxide. Retention times of atrazine and hydroxyatrazine were 7·3 min and 4·1 min, respectively. Standards containing atrazine (and metabolites, if necessary) were prepared from technical-grade material and were used whenever culture samples were analyzed.

At the end of the study, samples of the liquid were subjected to HPLC analysis to determine if remaining ^{14}C-materials were present as the parent atrazine, or as hydroxyatrazine. Atrazine alone was detected in these samples, indicating that remaining ^{14}C was associated with the undegraded compound.

2.8 Data comparison

Statistical comparisons of data for atrazine degradation were made using the analysis of variance procedure ($P = 0{\cdot}05$).[25] Comparisons between different environmental conditions were made based on total concentrations of atrazine degraded by the mixed culture.

To better understand the differences in carbon dioxide evolution data as a function of concentration and of liquid versus soil environment, the mineralization data were fitted to a three-half-order regression model.[26,27] The form of the model used was

$$P = S_0[1 - e^{-k_1 t - (k_2 t^2)/2}] + k_0 t,$$

where P was the percentage of atrazine mineralized, t the time after atrazine introduction and S_0 the percentage of carbon dioxide evolved from metabolism of atrazine, the initial substrate.[27] The parameter k_0 indicates zero-order mineralization of transformation products or initially unavailable atrazine, k_1 indicates first-order mineralization of atrazine, and k_2 indicates growth or increased degradative capacity of the mixed culture.[27] The coefficient of multiple determination (R^2) was calculated as an indication of quality of model fit to the data.

3 RESULTS

3.1 Soil system

Initial culture viability was evaluated at lower atrazine concentrations. Soil samples were placed in biometer flasks and treated with atrazine at 0·0014 mole kg^{-1} soil, as either technical grade or formulated material, and inoculated with the mixed culture. The mixed culture, was highly viable, as indicated by carbon dioxide evolution (Fig. 1), and rapid degradation of either formulated (79·4%) or technical grade (73·2%) atrazine.

Mineralization by the mixed culture was also evident when soil samples were inoculated with the mixed culture and treated with atrazine at concentrations of 0·046 and 0·23 mole kg^{-1} soil (Fig. 2). The mixed culture had mineralized 77·9% and 21·3% of atrazine in soil by 100 days at starting rates of atrazine of 0·046 and 0·23 mole kg^{-1} soil, respectively.

Soil inoculated with mixed culture was then treated with atrazine at 0·46 and 1·39 mole kg^{-1}-soil. There

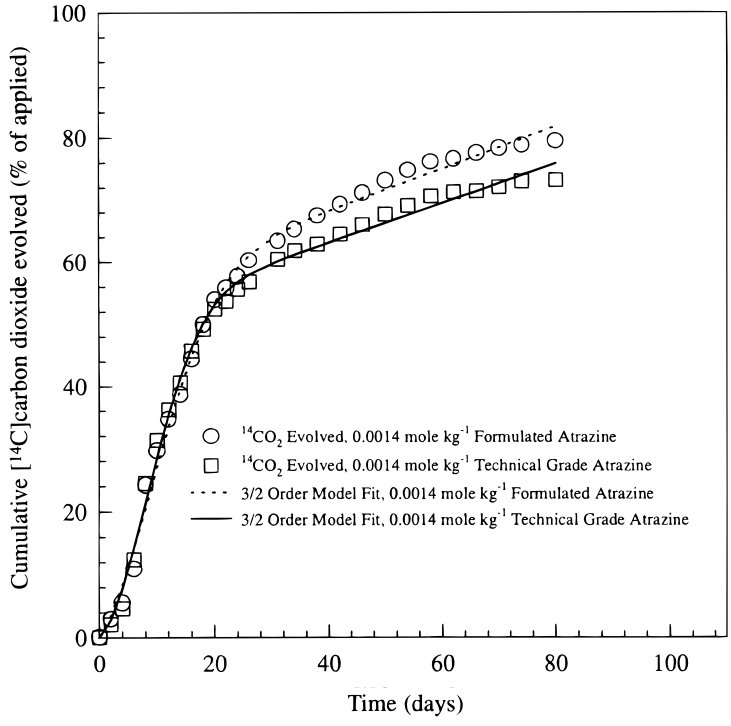

Fig. 1. Determination of mixed culture viability in soil at 0·0014 mole atrazine kg^{-1} soil. Mineralization of atrazine and the three-half-order model fit to atrazine mineralization data (measured as cumulative [^{14}C]carbon dioxide evolved) is shown.

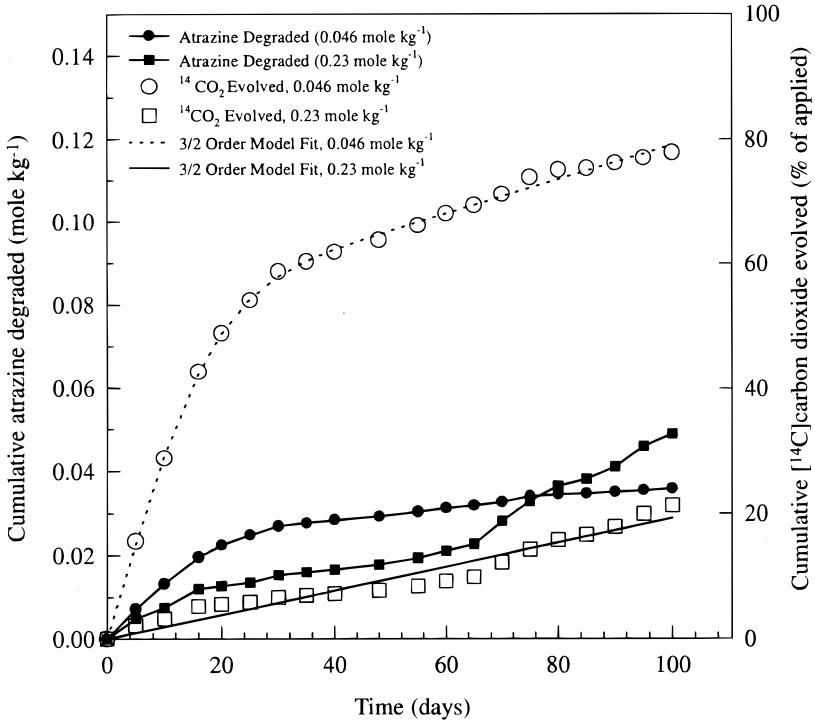

Fig. 2. Degradation of atrazine in soil at 0·046 and 0·23 mole kg^{-1} soil, presented as both concentration of atrazine mineralized and as percentage atrazine mineralized (as cumulative [^{14}C]carbon dioxide evolved). A three-half-order model fit to the latter is shown.

was little biodegradation of applied atrazine at these concentrations; after 40 days, 0·79% and 0·20% of the atrazine applied was degraded, respectively. At 40 days, soil samples were reinoculated to determine if biodegradation could be reinitiated, but no additional degradation of atrazine was detected.

Following unsuccessful soil biodegradation of atrazine at 0·46 and 1·39 mole kg^{-1} soil, a 0·46 mole kg^{-1}

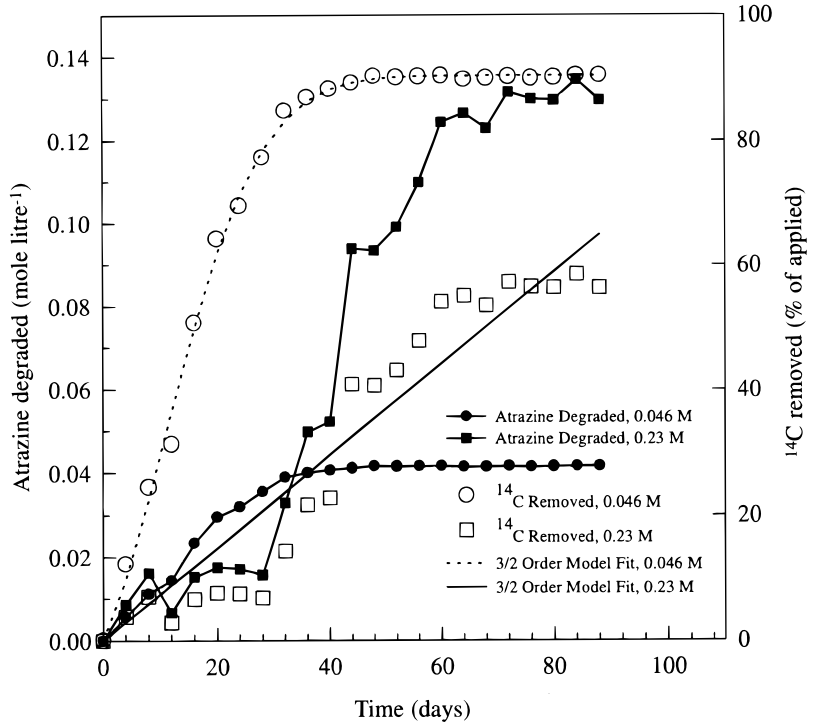

Fig. 3. Degradation of atrazine in liquid culture at 0·046 M and 0·23 M presented as concentration of atrazine degraded and as percentage of atrazine degraded (as ^{14}C removed from solution). A three-half-order model fit to the latter is shown.

soil treatment was evaluated with enhanced air recharge conditions. With the modified design, atrazine degradation increased slightly to 1·4% of the 0·46 mole kg^{-1} soil applied.

3.2 Liquid system

Mineralization of atrazine was evaluated in liquid samples, at either 0·23 M on 0·046 M concentrations. Maximum degradation of atrazine by the mixed culture occurred at 48 days for 0·046 M atrazine, with 90% degradation of the atrazine applied (Fig. 3). At 0·23 M atrazine, 56% of the atrazine applied was mineralized by the mixed culture in liquid media (Fig. 3), with degradation leveling off by 72 days. The percentage of atrazine mineralized in liquid samples exceeded that in soil samples at both atrazine concentrations: 90·4% versus 77·9% mineralized at 0·046 M and 56·4% versus 21·3% mineralized at 0·23 M, respectively. HPLC analysis of liquid samples at the end of the study indicated that no detectable metabolites were present in the media, although atrazine was present in concentrations indicating conservation of mass balance.

4 DISCUSSION

Bioremediation is an accepted technology for accelerating the rate of cleanup of contaminated soils and waters. Often the process is limited by a lack of adapted micro-organisms. We believe that the culture system that we have described fills a need as a bioaugmentation agent, as it is capable of degrading atrazine up to levels of 0·046 mole kg^{-1} soil, an amount far greater than that typically found in pesticide-contaminated soils ($7{\cdot}9 \times 10^{-5}$ to 1·9 mmole kg^{-1} soil.[6] Our data suggest that it can successfully mineralize atrazine at concentrations 20 times greater than the maximum concentrations found at agrochemical mixing-loading facilities. This work supports our earlier findings of mineralization of atrazine by this culture to concentrations of 0·56 mM in liquid culture and 0·14 mmole kg^{-1} in soil.[18] Moreover, it is capable of mineralizing atrazine in both soil and liquid environments, corresponding to solid- and slurry-phase bioremediation systems, respectively. While atrazine degradation at either the 0·46 or 1·39 mole kg^{-1} soil concentration was minimal, the amounts degraded exceeded most levels previously reported for other culture systems.[16,19,20]

Degradation studies with atrazine at 0·0014 mole kg^{-1} soil showed a rapid rate of degradation with either formulated or technical grade material. At starting concentrations of 0·046 and 0·23 mole kg^{-1} soil, 77·9% and 21·3% of soil-applied atrazine was degraded, respectively. However, in terms of the amount of atrazine degraded, the liquid system was more efficient than the soil system at either 0·046 M or 0·23 M treatment. The percentage of atrazine degraded in the liquid system was significantly greater ($P = 0{\cdot}05$) than that in soil at the 0·23 M concentration. Although not statistically significant, there was a trend for greater degradation of 0·046 M atrazine by the mixed culture in the liquid system. Also at the 0·046 M concentration, the time for maximum degradation in the liquid system was one-half of that in the soil system.

Decreased degradation in the soil system could be due to sorption, either direct herbicide–soil or indirect sorption where carrier, soil and pesticide interact. In either case the bioavailability of atrazine to the mineralizing population is reduced. Grimberg and Aitken[28] have shown that organic materials trapped in micelles, such as would be found within a herbicide carrier, are sometimes unavailable to the microbial population. They concluded that only materials in soil solution were available for degradation. It is not known what types of reaction occur between the soil and the herbicide carrier and how these interactions affect the behavior of the carrier materials. Direct sorption to soils has been shown to decrease the rate of degradation of flumetsulam.[29] Ogram *et al.*[30] report that sorbed (2,4-dichlorophenoxy)acetic acid (2,4-D) was not bioavailable for degradation by an introduced microbial population. Possibly indirect sorption of chemicals trapped within micelles may also limit their bioavailability and enhance their stability within contaminated soils.

Conversely, greater degradation in the liquid system most likely reflects a steady partitioning of atrazine from the formulated carrier to the aqueous phase of the liquid samples, a separation of oily components of the carrier material from the active population, and better air delivery to the degrading population. In essence, the formulated material is supplying a nearly saturating amount of chemical to the aqueous solution, eliminating chemical supply problems. Increased water content has been shown to enhance the degradation of DCPA (dimethyl tetrachloroterephthalate),[31] carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate)[32] and clomazone (2-(2-chlorophenyl) methyl)-4,4-dimethyl-3-isoxazolidinone) in soil systems.[33] In these cases better contact between the degrading population and the herbicide is made, generating conditions similar to those found in the liquid system. Another possible reason for greater degradation of atrazine in liquid samples is a greater initial population of degrading organisms per mass of medium in liquid versus soil system, which would also result in increased atrazine-micro-organism contact.

Atrazine was not degraded by the mixed culture at 0·46 and 1·39 mole kg^{-1} soil in inoculated soil and this did not take place on subsequent reinoculation.

Although the composition, of the herbicide carrier for formulated atrazine has not been reported, it many contain oils and other viscous materials. Soil samples with atrazine applied at the high concentrations (0·46 and 1·39 mole kg^{-1} soil) were completely coated by the formulated herbicide mixture. The low overall levels of degradation may have resulted from the inability of the micro-organisms to function in the oil-dominated system. The oil films could reduce diffusion across the air-soil interface, creating a barrier to oxygen and carbon dioxide diffusion to and from the soil/formulated atrazine mixture.[34,35] All of these factors could contribute to stability of the herbicide in these systems and the soils at mix-load sites.

Due to differences in reactor conditions, apparent growth of the culture on atrazine, and complexity of the carbon dioxide evolution data, a three-half-order model was used to estimate S_0, k_0, k_1 and k_2 parameters based on cumulative [^{14}C]carbon dioxide evolution data. The model has been previously used[18,27] to predict the same parameters based on cumulative [^{14}C]carbon dioxide evolution and provides insight into the microbial processes at work within the system.

A high quality of fit to carbon dioxide evolution data, as indicated by R^2 values (Table 1), was achieved with the three-half-order model at most concentrations. Fit S_0 parameters for all systems except the 0·23 M (mole kg^{-1} soil) atrazine application rate indicate that, at low concentrations, a portion of substrate was initially available for metabolism by the culture. The model fitted to the data from the 0·046 M liquid system indicates a much higher S_0 value than for the soil system at the same atrazine application rate. The high S_0 estimate is possibly due to the steady partitioning of atrazine from the carrier phase to the water phase, improving its bioavailability.

Near zero estimates for S_0 at the 0·23 M atrazine application rate indicate that atrazine was not directly available to the culture for degradation. The total atrazine (and herbicide carrier) concentration was five times greater than that of the 0·046 M system, causing a complex biodegradation scenario. The increased oily carrier phase concentration contributed to the pronounced lag phase in atrazine degradation and slow population growth of the culture, in both soil and liquid systems. As a result, the low S_0 values are likely due to the pronounced lag in atrazine degradation (Figs 2 & 3), and may be a greater reflection of the environment than the degradative capacity of the mixed culture.

For most of the atrazine degradation systems, the high k_0 value indicates mineralization of atrazine metabolites (Table 1), suggesting that atrazine is transformed to hydroxyatrazine prior to mineralization. While efforts to identify the atrazine-degrading organisms are ongoing, mixed culture and initial isolate cultures were evaluated for their genetic similarity to other atrazine-degrading organisms (see De Souza *et al.*[36]). Our mixed culture and one isolate were shown to contain the gene sequence for the *Ava*I DNA fragment (M. J. Sadowsky, pers. commun.) previously identified from *Pseudomonas sp.* strain ADP.[36] This sequence is responsible for the production of atrazine chlorohydrolase, an enzyme responsible for transformation of atrazine through the formation of the initial metabolite hydroxyatrazine.

The k_0 values support the idea that our mixed culture first dechlorinates atrazine, and then mineralizes the dechlorinated metabolite, hydroxyatrazine. In the liquid system with 0·046 M atrazine, we believe the near zero contribution to the model fit of hydroxyatrazine mineralization (k_0) is a system artifact. This suggestion is supported by the genetic analysis of the culture, which showed the presence of the *Ava*I gene responsible for the formation of hydroxyatrazine. We feel that the poor estimate of k_0 arises because the population conducts a nearly instantaneous utilization of the product, which

TABLE 1
Parameters from Carbon Dioxide Evolution Data Fitted to Three-Half-Order Model

Atrazine concentration (M)[a]	*Sample type*	*Three-half-order model parameter*[b] (±*SEM*)				
		S_0	k_0	k_1	k_2	R^2
0·0014	Soil	54·7 (±1·7)	0·34 (±0·03)	0·02 (±0·006)	0·008 (±0·001)	0·99
0·0014[c]	Soil	50·4 (±1·4)	0·32 (±0·03)	0·01 (±0·008)	0·012 (±0·002)	0·99
0·046	Soil	51·4 (±0·9)	0·28 (±0·01)	0·05 (±0·004)	0·004 (±0·0008)	0·99
0·046	Liquid	90·3 (±2·4)	—[d]	0·02 (±0·003)	0·004 (±0·0005)	0·99
0·23	Soil	—[d]	0·19 (±0·006)	—[d]	—[d]	0·94
0·23	Liquid	—[d]	0·74 (±0·03)	—[d]	—[d]	0·91

[a] Mole $litre^{-1}$ in liquid samples and moles kg^{-1} in soil samples.
[b] See Section 2.8 for description of parameters.
[c] Technical grade atrazine.
[d] Estimated to be zero by three-half-order regression analysis resulting in linear model fit.

causes the rapid rate of carbon dioxide loss from the liquid system. The model is unable to separate the processes, and the rapid rate of carbon dioxide loss is interpreted by the model as a high degree of initial availability of the substrate.

Moreover, in all systems, the fit k_1 and k_2 parameters are very low or zero (Table 1). Low estimates of k_1 (an indication of direct mineralization of the primary substrate atrazine), in conjunction with higher k_0 estimates, lend additional support to the idea that dechlorination of atrazine to hydroxyatrazine is needed prior to mineralization. This supports our suggestion that the zero estimate for k_0 in the 0·046 M liquid system is an artifact. In addition, the very low estimates of k_2 (estimation of biomass production) do not appear to represent system dynamics adequately.

Very low estimates of k_2 would suggest that atrazine contributes little to biomass production. We feel that the low k_2 values reflect the high overall evolution of [^{14}C]carbon dioxide, as this model assumes pesticide substrates to be used as a carbon source. However, in our case the formation of [^{14}C]carbon dioxide is an indication of ring catabolism to serve as nitrogen source.[17,21,37,38] The oxidation status of ring carbons of atrazine limits their use in biomass production, creating a situation where catabolism of atrazine results in the evolution of ring carbon as carbon dioxide[37] and release of nitrogen in the form of ammonia.[39] The result of this catabolic pathway is the evolution of [^{14}C]carbon dioxide and little incorporation of ^{14}C into biomass. We feel that this process creates the low k_2 estimates within the three-half-order model. That is, the fitted parameters are developed corresponding to the [^{14}C]carbon dioxide evolution curve. The difference between the starting level of atrazine and the amount of carbon dioxide evolved is assumed to be incorporated into biomass. Our mixed cultures are complex systems in which the target substrate serves as a nitrogen source, while a glycerol supplement serves as the carbon source. One recent study has shown that amino-nitrogen at the 4 and 6 position of atrazine was directly incorporated by the degrading population, while ring nitrogen was excreted into the medium as ammonia.[40] In our mixed culture, it is probable that peripheral members of the mixed culture are serving as a sink for a portion of the atrazine nitrogen converted by the degrading population but do not take up ^{14}C from atrazine. This could result in lower apparent population growth, as suggested by the model.

Degradation of atrazine (0·046 and 0·23 M (mole kg^{-1}-soil)) was incomplete as evidenced by [^{14}C]carbon dioxide evolution. In the 0·046 M liquid system, about 90% of applied atrazine was degraded at the termination of the study. Biomass production was not directly measured due to problems associated with cell extraction from the contaminated soil and liquid samples. However, a theoretical biomass can be calculated using the approach taken by Turco and Konopka.[41]

Using the percentage bacterial composition values suggested by Luria,[42] 50% carbon 14%, and 3% phosphorus (dry weight), and the use of atrazine (32% nitrogen) as the sole nitrogen source, the amount of atrazine degraded in the 0·046 M liquid system (0·50 g atrazine per sample) would have released 0·14 g of nitrogen. Based on nitrogen release, this degradation could support approximately 1 g atrazine per biomass sample. These same assumptions applied to degradation of atrazine in the 0·23 M liquid system (2·4 g atrazine per sample) would have resulted in 0·45 g nitrogen released and formation of 3 g biomass per sample. In other studies we have observed a 100-fold increase in cell number (direct count) in 10 days with atrazine (0·23 mM) as the sole source of nitrogen in a minimal basal salts medium. This shows the ability of atrazine, as the sole nitrogen source, to support cell production.

Using the theoretical carbon content of cells (50%), five times more carbon than was applied to the media as glycerol was required for the observed degradation of atrazine as nitrogen source. We suggest that some of the formulated herbicide carrier material was degraded as a carbon source by the mixed culture when utilizing the nitrogen liberated during the mineralization of atrazine. As a result, we assume that carbon supply is not limited in these systems and did not contribute to the incomplete degradation of atrazine in the liquid systems. However, in soils from contaminated sites, carbon may be limited and add to the stability of the contaminant.

When we assume that degradation of atrazine as a nitrogen source results in biomass production (and using nutrient values stated earlier), the mineralization of 56% of the available atrazine (0·23 M system) would result in the utilization of greater than 100% of the phosphorus supplied with the nutrient solution. The depletion in available phosphorus could result in a low energy status of the atrazine degraders, and a subsequent slowdown of enzyme systems required for degradation of atrazine.

The depletion of phosphorus could also explain the low percentage of degradation reported in the soil systems. The soil systems were not supplemented with phosphorus, and degrading and peripheral microorganisms would be dependent on release of phosphorus from soil for biomass production. The slow release of soil phosphorus to the soil solution probably results in incomplete degradation of atrazine in the soil systems. This points out the need to consider all nutrient needs when considering a bioremediation system and why some chemicals appear to be stable in the soils at mix-load sites.

In all of the 0·046 M and 0·23 M systems evaluated, overall atrazine degradation was very high. However, in all cases, degradation was incomplete. Possible reasons for this include: (1) a buildup of toxic end products

resulting from degradation of the carrier material of the formulated atrazine, (2) retention of atrazine in the carrier phase, (3) interactions between soil and atrazine or the degrading population or (4) depletion of a primary nutrient source in the liquid media.

The carrier retention and toxicity explanations seem unlikely, as we have degraded three times more atrazine at the 0·23 M level than in the 0·46 M liquid system. Toxic effects would most likely have occurred in the 0·046 M liquid system as well, but were not seen, as percentage degradation was higher in this system than in the 0·0014 mole kg^{-1} soil system. Sorption interactions involving atrazine probably contributed to incomplete degradation in soil samples, however, at the 0·046 and 0·23 mole kg^{-1} soil application rates; this contribution would probably not be great.

Bearing in mind the improbability of toxicity and carrier retention problems, we suggest that the primary reason for incomplete degradation in the soil and liquid system was mineral nutrient limitation. However, we do feel that a small portion of the incomplete degradation could be attributed to slow diffusion from the carrier to the water phase (like the 0·046 M system). There should be a large portion of applied atrazine still available to the degrading microbial population.

These findings indicate that, while the mixed culture is capable of degrading great quantities of atrazine, it can still be limited by nutrient supply, either nitrogen (atrazine) or phosphorus (either supplemented or native soil sources). Carbon source does not appear to be limiting when degrading formulated atrazine, as the carrier can apparently be utilized by the mixed culture. When considering the use of a microbial population as a remediation tool for high concentrations of pesticide, it is important to consider the available phosphorus supply, as well as other nutrients.

Better degradation of atrazine by the mixed culture in liquid versus soil samples was observed, both in terms of overall atrazine degraded and the time required for degradation. The three-half-order model is a valuable model for prediction of dynamics of pesticide degradation. Data from the three-half-order model support dechlorination of atrazine and formation of readily degraded hydroxyatrazine. However, we suggest care in using the model where the pesticide can serve as a nutrient source other than carbon. We feel that this bias caused a marked underestimation in the biomass component of the model (k_2) when applied to pesticide substrates catabolized as a nitrogen source.

With evidence that the mixed culture enhances atrazine degradation at elevated concentrations in liquid and soil samples, and the possibility that the mixed culture population is adaptive to shifts in atrazine concentration, we suggest that the mixed culture could be utilized in *ex-situ* bioremediation of soil and water contaminated with atrazine. This would include ground water, well water or rinse water from equipment cleaning, which could originate from an agrochemical mixload site. The use of the mixed culture in a slurry-phase remediation system also shows great promise.

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REFERENCES

1. *Weed Science Society of America Herbicide Handbook*, 6th edn. Weed Science Society of America, Champaign, IL, 1989, pp. 17–20.
2. Ritter, W. F., Pesticide contamination of ground water in the United States—a review. *J. Environ. Sci. Health*, **B25** (1990) 1–29.
3. Junk, G. A., Spalding, R. F. & Richard, J. J., Aerial, vertical and temporal differences in ground water chemistry: II. Organic constituents. *J. Environ. Qual.*, **9** (1980) 479–83.
4. Habecker, M. A., Environmental contamination at Wisconsin pesticide mixing/loading facilities: Case study, investigation and remedial action evaluation. *Wisc. Dept. Agriculture, Trade and Consumer Prot., Ag. Res. Mgmt Rpt*, 1989.
5. Goetsch, W. D., Kirbach, G. C. & Black, W. F., Pesticides in well water and ground water at agrichemical facilities in Illinois: An initial investigation. In *Agrichemical Facility Site Contamination Study*, Illinois Dept Agriculture, 1993.
6. Krapac, I. G., Roy, W. R., Smyth, C. A. & Barnhardt, M. L., Occurrence and distribution of pesticides in soil at agrichemical facilities in Illinois. In *Agrichemical Facility Site Contamination Study*, Illinois Dept Agriculture, 1993.
7. Koybayashi, H. & Rittmann, B. E., Microbial removal of hazardous organic compounds. *Environ. Sci. Technol.*, **16** (1982) 170A–183A.
8. Forsyth, J. V., Tsao, Y. M. & Bleam, R. D., Bioremediation: When is augmentation needed? In *Bioaugmentation for Site Remediation*, ed. R. E. Hinchee, J. Fredrickson and B. C. Alleman. Battelle Press, Columbus, Ohio, 1995, pp. 1–14.
9. Risatti, J. B. & Zagula, S., Potential cost effective technologies for remediation of Illinois agrichemical facilities. In *Agrichemical Facility Site Contamination Study*, Illinois Dept Agriculture, 1993.
10. Kaake, R. H., Roberts, D. J., Stevens, T. O., Crawford, R. L. & Crawford, D. L., Bioremediation of soils contaminated with the herbicide 2-*sec*-butyl-4,6-dinitrophenol (dinoseb). *Appl. Environ. Microbial.*, **58** (1992) 1683–9.
11. Shin, C. Y. & Crawford, D. L., Biodegradation of trinitrotoluene (TNT) by a strain of *Clostridium bifermentans*. In *Bioaugmentation for Site Remediation*, ed. R. E. Hinchee, J. Fredrickson and B. C. Alleman. Battelle Press, Columbus, Ohio, 1995, pp. 57–69.
12. Edgehill, R. U. Removal of pentachlorophenol from soil by *Arthrobacter* strain ATCC 33790. In *Bioaugmentation for Site Remediation*, ed. R. E. Hinchee, J. Fredrickson and

B. C. Alleman. Battelle Press, Columbus, Ohio, 1995, pp. 85–90.
13. Baud-Grasset, F. & Vogel, T. M., Bioaugmentation: Biotreatment of contaminated soil by adding adapted bacteria. In *Bioaugmentation for Site Remediation*, ed. R. E. Hinchee, J. Fredrickson and B. C. Alleman. Battelle Press, Columbus, Ohio, 1995, pp. 39–48.
14. Behki, R. M. & Khan, S. U., Degradation of atrazine by *Pseudomonas*: N-dealkylation and dehalogenation of atrazine and its metabolites. *J. Agric. Food Chem.*, **34**, (1986) 746–9.
15. Khan, S. U. & Behki, R. M., Effects of *Pseudomonas* species on the release of bound ^{14}C residues from soil treated with [^{14}C]atrazine. *J. Agric. Food Chem.*, **38** (1990) 2090–3.
16. Behki, R., Topp, E., Dick, W. & Germon, P., Metabolism of the herbicide atrazine by *Rhodococcus* strains. *Appl. Environ. Microbiol.*, **59** (1993) 1955–9.
17. Mandelbaum, R. T., Wackett, L. P. & Allan, D. L., Mineralization of the *s*-triazine ring of atrazine by stable bacterial mixed cultures. *Appl. Environ. Microbiol.*, **59** (1993) 1695–1701.
18. Assaf, N. A. & Turco, R. F., Accelerated biodegradation of atrazine by a microbial consortium is possible in culture and soil. *Biodegradation*, **5** (1994) 29–35.
19. Mougin, C., Laugero, C., Asther, M., Dubroca, J., Frasse, P. & Asther, M., Biotransformation of the herbicide atrazine by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.*, **60** (1994) 705–8.
20. Yanze-Kontchou, C. & Gschwind, N., Mineralization of the herbicide atrazine as a carbon source by a *Pseudomonas* strain. *Appl. Environ. Microbiol.*, **60** (1994) 4297–302.
21. Radosevich, M., Traina, S. J., Hao, Y-L. & Tuovinen, O. H., Degradation and mineralization of atrazine by a soil bacterial isolate. *Appl. Environ. Microbiol.*, **61** (1995) 297–302.
22. Wolfe, H. R., Staiff, D. C., Armstrong, J. F. & Comer, S. W., Persistence of parathion in soil. *Bull. Environ. Contam. Toxicol.*, **10** (1973) 1–9.
23. Yanze-Kontchou, C. & Gschwind, N., Mineralization of the herbicide atrazine in soil inoculated with a *Pseudomonas* strain. *J. Agric. Food Chem.*, **43** (1995) 2291–4.
24. Gan, J., Becker, W. C., Koskinen, W. C. & Buhler, D. D., Degradation of atrazine in two soils as a function of concentration. *J. Environ. Qual.*, **25** (1996) 1064–72.
25. Steel, R. G. D. & Torrie, J. H., *Principles and Procedures of Statistics*, 2nd edn. McGraw-Hill Book Co., New York, 1980.
26. Brunner, W. & Focht, D. D., Deterministic three-half-order kinetic model for microbial degradation of added carbon substrates in soil. *Appl. Environ. Microbiol.*, **47** (1984) 167–72.
27. Moorman, T. B. & Harper, S. S., Transformation and mineralization of metribuzin in surface and subsurface horizons of a Mississippi delta soil. *J. Environ. Qual.*, **18** (1989) 302–6.
28. Grimberg, S. J. & Aitken, M. D. Biodegradation kinetics of phenanthrene solubilized in surfactant micelles. In *Microbial Processes for Bioremediation*, ed. R. E. Hinchee, C. M. Vogel and F. J. Brockman. Battelle Press, Columbus, Ohio, 1995, pp. 59–66.
29. Lehmann, R. G., Miller, J. R., Fontaine, D. D., Laskowski, D. A., Hunter, J. H. & Cordes, R. C., Degradation of a sulfonamide herbicide as a function of soil sorption. *Weed Res.*, **32** (1992) 197–205.
30. Ogram, A. V., Jessup, R. E., Ou, L. T. & Rao, P. S. C., Effects of sorption on biological degradation rates of (2,4-dichlorophenoxy)acetic acid in soils. *Appl. Environ. Microbiol.*, **49** (1985) 582–7.
31. Choi, J. S., Fermanian, T. W., Wehner, D. J. & Spomer, L. A., Effect of temperature, moisture and soil texture on DCPA degradation. *Agron. J.*, **80** (1988) 108–13.
32. Shelton, D. R. & Parkin, T. B., Effect of moisture on sorption and biodegradation of carbofuran in soil. *J. Agric. Food Chem.*, **39** (1991) 2063–8.
33. Mervosh, T. L., Sims, G. K. & Stoller, E. W., Clomazone fate in soil as affected by microbial activity, temperature and soil moisture. *J. Agric. Food Chem.*, **43** (1995) 537–43.
34. Downing, A. L. & Truesdale, G. A., Some factors affecting the rate of solution of oxygen in water. *J. Appl. Chem.*, **5** (1955) 570–81.
35. Hawke, J. G. & Alexander, A. E., The influence of surface-active compounds upon the diffusion of gases across the air–water interface. In *Retardation of Evaporation by Monolayers: Transport Processes*, ed. V. K. LaMer. Academic Press, New York, 1962, pp. 67–73.
36. De Souza, M. L., Sadowsky, M. J. & Wackett, L. P., Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: Gene sequence, enzyme purification and protein characterization. *J. Bacteriol.*, **178** (1996) 4894–900.
37. Cook, A. M., Biodegradation of *s*-triazine xenobiotics. *FEMS Rev.*, **46** (1987) 93–116.
38. Mulbry, W. & Kearney, P. C., Degradation of pesticides by micro-organisms and the potential for genetic manipulation. *Crop Prot.*, **10** (1991) 334–46.
39. Harris, R. F. & Adams, S. S., Determination of the carbon-bound electron composition of microbial cells and metabolites by dichromate oxidation. *Appl. Environ. Microbiol.*, **37** (1979) 237–43.
40. Bichat, F., Sims, G. K. & Mulvaney, R. L., Fate of ring-nitrogen during microbial decomposition of atrazine. In *Agronomy Abstracts*. American Society of Agronomy, Madison, WI, 1996, p. 238.
41. Turco, R. F. & Konopka, A., Biodegradation of carbofuran in enhanced and non-enhanced soils. *Soil Biol. Biochem.*, **22** (1990) 195–201.
42. Luria, S. E., In *The Bacteria*, Vol. 1, ed. I. C. Gunsalus and R. Y. Stanier. Academic Press, New York, 1960, p. 15.